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(54) Title: COMPOSITION AND METHOD FOR TREATING AN ENCEPHALOMYELOPATHIC, DEMYELINATING OR AUTOIMMUNE DISEASE

(57) Abstract

Method and pharmaceutical composition for the treatment of an encephalomyelopathic, demyelinating or autoimmune disease, the composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons.

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COMPOSITION AND METHOD FOR TREATING AN ENCEPHALOMYELOPATHIC, DEMYELINATING OR AUTOIMMUNE DISEASE.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for treating encephalomyelopathic, demyelinating or autoimmune diseases, particularly multiple sclerosis. The invention takes advantage of discoveries concerning the ability of a component or components of bee venom of molecular weight cut-off of about 12,000 Daltons to inhibit or reverse symptoms associated with an encephalomyelopathic disease model.

BACKGROUND OF THE INVENTION

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic nervous system disease of considerable clinical importance, found in males and females, with onset usually beginning between age 20 and 40. Over 250,000 individuals suffer from the disease in the U.S., about two-thirds of them women; worldwide, there are about 1 million cases of MS Steinman and Conlon, 1995, Bio/Technology 13:118-119). It is the commonest demyelinating disorder of the brain and spinal chord, characterized by areas of demyelination or "plaques." MS can have a subtle onset, and is characterized by remissions and relapses, but its course becomes relentlessly progressive in about one-third of patients. Although the specific cause of MS is unknown, considerable evidence supports an immunological component in the pathogenesis of the disease: perivascular mononuclear cellular infiltrates are found in MS lesions, macrophage-dependent phagocytosis of myelin in the CNS

white matter is observed, T lymphocytes have been identified in the perivascular cuff and adjacent white matter (Rose et al., 1991, Clin. Immunol. and Immunopathol. 59:1-15), and cerebrospinal fluid in MS contains oligoclonal populations of immunoglobulins *(see* Schwartz, 1993, In *Fundamental Immunology, Third Edition,* William E. Paul (ed.), Raven Press: New York, pp. 1033-1097, 1079). Symptoms of the disease include some degree of paralysis, incoordination, loss of sensation, tremor, nystagmus, blindness, disturbance of speech, and bowel and bladder incontinence. The array and severity of symptoms depend on the site and extent of the lesion.

The target of inflammation associated with MS has been elusive, but evidence indicates a reaction to a part of the myelin sheath, particularly of the nerves of the brain, retina, and spinal chord. It is generally believed that MS is an example of autoimmune disease, a category that includes rheumatoid arthritis, juvenile onset - diabetes, systemic lupus erythematosus, and thyroiditis. Genetics and the environment influence susceptibility to autoimmune disease, and MS is no exception. In MS, nearly 75 % of patients have HLA-DR2 haplotype (Steininan and Conlon, *supra),* and this haplotype is associated with recognition of antigenic determinants derived from the central nervous system (CNS), such as myelin basic protein (MBP) and proteolipid protein (PLP) (Schwartz, *supra).* It is also well known that relapses of MS are often proceeded by viral or bacterial infection (Steinman and Conlon, *supra),* and bacterial superantigen can induce relapse in an experimental model of MS *(ibid., see* Racke et al., 1994, J. Immunol. 152:2051).

Previously, attempts were made to treat MS with steroid therapy. Actually, treatment strategies for MS have included, but are not limited to, adenocorticotrophic

hormone (ACTH) and corticosteroids (antiinflammatory), azathioprine and cyclophosphamide (cytotoxins), total lymphoid irradiation, plasma exchange (plasma-pheresis), and cyclosporine A, interferons, cytokines, and monoclonal antibody therapies (immune-modulation). Recent efforts, however, have focused on more direct, and effective, therapies. Recombinant and natural beta interferon (IFN-B) has been found in clinical trials to reduce the number of active lesions of MS as detected by magnetic resonance imaging (MRI), to increase the likelihood of remaining exacerbation-free, to decrease the incidence of flare-ups, and delay the progression of disability. However, it appears that beta interferon will be more effective in treating MS in the relapsing-remitting stage. Moreover, this drug manifests adverse side effects, including flu-like symptoms and site injection reactions.

In addition to beta-interferon, other strategies are also being pursued for treating MS. One group employs a synthetic random copolymer of tyrosine, glutamate, alanine, and lysine, called Cop-I (more commonly referred to as copolymer I or *Copaxone®* (TEVA Pharmaceuticals, USA)), to apparently block T-cell recognition of MBP. In a 250-patient trial, the relapse rate of placebo patients was much higher than in individuals receiving the drug. Other inhibitors of the immune response to MBP are also being developed. These include, but are not limited to, peptide ligands, MHC antagonists, and altered T cell receptors; one of these reverses the rodent model of MS after a single injection. Presumably, this treatment works by inactivating, or tolerizing, autoimmune T cells. Yet another group is feeding MBP to induce tolerance, a technique that has demonstrated some success with DR2-negative, but not DR2-positive, patients. Attempts at treatment by immunization with T cell receptor fragments,

and the use of monoclonal antibodies against CD4 (a T cell marker), T cell adhesion molecules, or the T cell receptor (or a specific class of T cell receptor), are also proceeding with modest success.

EAE: An Animal Model of Multiple Sclerosis

Knowledge of the pathogenesis of autoimmune diseases has been derived from the study of animal models. Experimental allergic (or autoimmune) encephalomyelitis (EAE) is considered a model of multiple sclerosis. However, EAE is an acute model for a chronic disease. Nevertheless, aspects of EAE provide insight into the chronic disease through upregulation of response to autoantigen (Heber-Katz, 1993, Intern. Rev. Immunol. 9:277-285). The first incidences of EAE (resulting from the Pasteur rabies vaccine that was prepared from rabbit brain) led to the discovery of myelin components as the antigenic agents. Indeed, T cells directed to specific myelin components, such as myelin basic protein (MBP), are found in MS patients (Heber-Katz, 1994, "Is Experimental Allergic Encephalomyelitis a Model of Multiple Sclerosis?", In *Autoimmunity. Physiology and Disease,* Coutinho and Kazatchkine (eds.), Wiley-Liss: New York, p. 353). It has been found that any myelin-associated antigen, such as proteolipoprotein (PLP), myelinassociated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG), can cause EAE *ibid.)*.

The relapsing course of MS has been particularly difficult to reproduce in animal models, particularly EAE, which is a monophasic and acute model. In the past decade, however, chronic (relapsing) forms of EAE have been developed (Alvord et al., 1985, Neurochemical Pathology 3:195-214; Bourdette et al., 1986, Neurochemical Pathology 4:1-9; Fallis and McFarlin, 1989,

J. Immunol. 143:2160-2165; Driscoll et al., 1982, J. lmmunol. 128:635-638; Traugott et al., 1982, Cell. Immunol. 68:261-275).

Apitherapy: Folk Remedy

A group of individuals (notably the American Apitherapy Society, Inc.) advocate treating conditions such as arthritis and MS with bee stings, have published explicit therapeutic protocols, and have reported in publications of the American Apitherapy Society that bee sting therapy effectively treats MS. In addition, positive therapeutic results attributed to bee venom therapy have bean reported in the popular media, including television news programs. However, bee venom therapy has not undergone any scientific evaluation, nor have the alleged positive results been reported in peer reviewed scientific journals; thus, any reported positive results are suspect because of the limited patient population tested, the failure to control for a placebo effect, and the fact that many of the diseases for which apitherapy has reportedly been successful, notably MS, are naturally remitting-relapsing disease, in which remission can occur spontaneously. The unscientific "studies" conducted to date fail to employ any controls of these variables. The scientific and medical community regards these reports with skepticism or outright disbelief (*see* Habermann, 1972, Science 177:314-322). Furthermore, bee stings are unpleasant and elicit allergic reactions in sensitive individuals that are potentially lethal.

Bee stings have long been regarded in folk medicine as effective treatments for any number of chronic inflammatory diseases, including arthritis, neuritis, and fibromyositis. "`Old wives tales' tracing back to antiquity advocate bee preparation for conditions as

varied as baldness, toothache, kidney stones, eye diseases, and gout" (Banks and Shipolini, 1986, "Chemistry and Pharmacology of Honey-bee Venom," In *Venoms of the Hymenoptera. Biochemical, Pharmacological and Behavioural Aspects,* T. Piek (ed.), Academic Press: London, p. 329). Anecdotal reports indicate benefit of bee venom on pathologic cardiovascular changes, such as hypertension ("The Treatment of Angiocardiopthy with BVT", Bee Informed, Spring 1994, p. 1). However, as noted above, these reports tend to be carried in publications of minimal or no scientific merit *(e.g.,* Bee Informed, the American Apitherapy Society Newsletter), and report conclusions that have no statistical basis and lack appropriate controls. The information tends to be dominated by anecdotal reports.

Recently, the Multiple Sclerosis Association of America announced plans to fund a clinical study of the effects of bee venom therapy on MS. However, the organization itself characterized apitherapy as "... an unbelievable movement throughout the MS community with little to no scientific evidence" ("MS Association to Study Bee Venom," American Bee Journal, April 1995, p. 233).

Components of Bee Venom

The composition of Hymenoptera venoms have been extensively reviewed (Piek, 1986). In general, Hymenoptera venoms are mixtures of biogenic amines, peptides and proteins. Honey bee *(Apis mellifera)* venom contains a number of important pharmaceutically active agents. These include biogenic amines, amino acids, oligopeptides, peptides, enzymes, and other molecules. The components of honey bee venom are summarized in Table 1:

Table 1. Compositions of honey bee venom.

COMPONENT	MOL. WT.	% DRY WEIGHT	TOMCITY	ALLERGENI-CITY
low molecular wt. substances	up to 1,000	25	low	none
Pheramones (volatile)		-		
Histamine	111	1	local	none
Dopamine	153	1	-	-
Noradrenalilne	169	1	-	-
Amino-Acids	100-200	1	-	-
Oligopeptides	200-1,000	14	-	-
Phospholipids	100-400	5	-	-
Carbolydrates	180	2	-	-
Larger Peptides	1,000-5,000	60	marked	mostly none
Melittin	2,840	50	membrane poison	+
Apamine	2,038	2	neurotoxin	-
MCD-Peptide	2,593	2	Histamine-liberator	-

Tertiapine	2,000	0.1	Histamine-liberator	-
Secapine	ca. 2,600	0.5	?	-
Cardiopep	?	0.7	+ chronotrop + inotrop	-
Enzymes	10,000 200,000	15	low (exception PLA_2)	high
Phospholipase A_2	19,000	12	membrane poison	++++
Lysophospholi-pase	22,000	1		?
Hyaluronidase	45,000-50,000	2	spreadingfa ctor	+++
Acid Phosphatase	49,000	1		++
Alpha-Glucosidase	170,000	1		?
Esterases	?	1		?
Others				
Adolapin	11,092	1	analgetic,	?

			blockade of arachidonic acid metabolism	
Protease Inhibitor	9,000	0.8		?
Allergen C	105,000	1	?	+

From Mueller, 1990, "Insect Venoms", Insect Sting Allergy. Clinical Picture, Diagnosis and Treatment, *Gustav Fischer: Stuttgart, p. 23.*

Closely related to the honey-bees are bumble-bees, and bumble-bee venom is very similar to that of the honey-bee (Mueller, *supra,* p. 26; Piek, 1986, "Venoms of Bumble-bees and Carpenter-bees, " In *Venoms of the Hymenoptera. Biochemical, Pharmacological and Behavioural Aspects,* T. Piek (ed.), Academic Press: London, p. 417). However, bumble-bee venom lacks melittin, and instead contains a unique structural class of peptides that have similar biological properties: the bombolitins, which lyse erythrocytes and liposomes, induce histamine release, and stimulate phospholipase A_2 (Hoffman, 1982, J. Allergy Clin. Immunol. 69:139; Piek, *supra;* Argiolas and Pisano, 1985, J. Biol. Chem. 260:1437-1444; Argiolas and Pisano, 1983, J. Biol. Chem. 258:13697-113702).

Carpenter-bee venom also contains histamine, and its properties demonstrate the presence of a component that induces effects mediated by bombolitin and melittin (Piek, *supra,* p. 422-23).

The venom of the vespidae (social wasps) are similar to each other, and, though not as well investigated as honey-bee venom, contain many of the same or similar components. Lower molecular weight peptides, amino acids, and biogenic amines -- notably histamine -- make up about a quarter of the dry weight of vespid venoms, while higher molecular weight proteins, notably phospholipase and hyaluronidase, make up the remaining quarter of venom dry weight (Mueller, *supra,* p. 27; King et al., Mol. Immunol. 20:297-308). The components of vespid venoms are summarized in Table 2:

Table 2. Compositions of vespid venoms.

COMPONENT	MOL.WT.	% DRY WEIGHT	FOUND IN*	ALLERGENIC ACTIVITY
low molecular weight substances	up to 1,000			-
Pheramones (volatile)		-		-
Histamine	111	3-6	V, D, H, P	-
Serotonin	176	1	V, D, H, P	-
Acetylcholine	182	1	H	-
Catecholamines	150-200	1	V, D, H, P	-
Carbohydrates	180		H, P	-
Amino-Acids	100-200		V, D, H, P	-
Polyamines			V, D, H, P	
Peptides	1,000-6,000		V, D, H, P	-
Kinins	1,000-3,000		V, D, H, P	-
Mastoparan	1,500		V, H, P	-
Chemotactic peptide	1,500		H	-

Haemolysin	6,000		V, H	+
Enzymes				
Phospholipase (A + B)	35,000	6-14	V, D, H, P	+++
Hyaluronidase	45,000	1-3	V, D, H, P	+++
Acid Phosphatase	?		V, D	+
Alkaline Phosphatase	?		V	+
Protease	?		V, D, H	++
DNase	?		V, P	?
Cholinesterase	?		V	?
Histidindecarboxylase	?		V, D	?
Saccharidase	?		H	?
Others				
Antigen 5	25,000	5-10	V, H, D, P	+++
Vmac 1	97,000		V, D	+
Vmac 3	39,000		V	+

* (V = vespula; D = dolichovespula; H = hornet; P = polistes)

From Mueller, *supra*.

WO 90/03178 discloses an anti-inflammatory and analgesic compound derived from ant venom and suitable for treating auto-immune diseases. The compound may be purified by a method involving the steps of a) sequentially filtrating an aqueous solution of the raw venom through two ultrafiltration membranes having fraction molecular weights of 10,000 and 1,000, respectively, b) subjecting the resulting venom fraction having a molecular weight of less than 1,000 to heat treatment, c) removing the insoluble parts by filtration, and d) sequential treatment on three chromatographic columns filled with anion exchange resin, hydrophilic vinyl resin and cation exchange resin, respectively.

The citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

SUMMARY OF THE INVENTION

In its broadest aspect, the present invention is directed to a method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease, more particularly, an autoimmune encephalomyelopathic disease, and preferably an encephalomyelopathic disease such as multiple sclerosis, comprising the administration to a subject suffering from the disease a therapeutically effective amount of a component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding the compound of claim 1 of WO 90/03178. Preferably, the treatment is effective to prevent further deterioration of a symptom of the disease. Preferably, the hymenoptera venom is apid venom or vespid venom. In a specific embodiment, the venom is honey-bee *(Apis mellifera)* venom.

Preferably, the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is effective to inhibit or reverse a symptom of the disease, or the said component has a positive response in an Experimental Allergic Encephalomyelitis (EAE) model, or the said component is capable of blocking T-cell response of a myelin-associated antigen.

In a specific embodiment, *infra,* a low molecular weight preparation of hymenoptera venom comprises all of the components of hymenoptera venom having a molecular weight of less than about 12,000 Daltons. In a specific embodiment, these components are present in approximately the same proportion as found in natural hymenoptera venom, or as found in a low molecular weight fraction of hymenoptera venom prepared by liquid size fractionation.

In a specific embodiment, the component or components of hymenoptera venom are prepared by recombinant technologies. Furthermore, the component of hymenoptera venom may be prepared by chemical or enzymatic peptide synthesis, e.g. as described in International patent application No. WO89/06656.

In a specific embodiment, *infra,* a component of honey-bee venom having a molecular weight of less than about 12,000 Daltons is prepared by fractionating whole honey-bee venom into fractions having a molecular weight greater than about 12,000 Daltons and less than about 12,000 Daltons using an ultrafiltration cell having a 10 kilo-Dalton cut-off membrane.

According to the invention, any effective route of administration of the low molecular weight venom component can be employed. For example, the component of

hymenoptera venom having a molecular weight of less than about 12,000 Daltons can be administered parenterally. Routes of parenteral administration include, but are by no means limited to, intravenous, subcutaneous, intradermal, intraperitoneal, intramuscular, intraarterial, and intraventricular. Alternatively, the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons can be administered orally, nasally, transmucosally, transdermally, or by inhalation (pulmonary administration).

In a specific embodiment, the amount of the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons corresponds to the amount present in from about 5 μg/kg/day of whole hymenoptera venom to about 500 μg/kg/day of whole hymenoptera venom. More preferably, the amount of the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons corresponds to the amount present in from about 25 μg/kg/day of whole hymenoptera venom to about 150 μg/kg/day of whole hymenoptera venom.

In a preferred aspect of the invention, the subject in whom treatment is effected is a human, and the disease is multiple sclerosis.

The invention further contemplates administration of another agent to modulate the effects or side effects of the low molecular weight fraction of bee venom. In one aspect, the method of the invention may further comprise administering an anesthetic or an analgesic. In another embodiment, the invention contemplates administering an anti-inflammatory drug. The anti-inflammatory drug may be a non-steroidal anti-inflammatory drug, a steroid, a non-inflammatory cytokine (for example, interferon-β), or an inhibitor of an inflammatory cytokine.(Reisman RE, IN:

Immunology and Allergy Clinics of North America, Immunotherapy of IgE-mediated Disorders, Greenberger PA, editor, W.B. Saunders Co., Philadelphia, PA, v 12, p 85-94, February 1982.)

A major advantage of the present invention is that the low molecular weight fraction selected according to the invention does not contain many of the strongest allergenes present in hymenoptera venom. Thus, the two strongest allergenes of apid venom, phospholipase and hyaluronidase, cf. Table 1, are not present in a low molecular weight fraction of apid venom according to the invention, and likewise the three strongest allergenes of vespid venom, phospholipase, hyaluronidase and Antigen 5, cf. Table 2, are not present in a low molecular weight fraction

The invention further relates to a method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract.

The invention further relates to a method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons excluding compositions,

which are derived from ant venom extract from a *pseudomyrmex triplarinus* ant.

The invention further relates to a method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons and more than about 1,000 Daltons.

The invention further relates to a method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *pseudomyrmex triplarinus* ant, and which contains one or more components having an apparent molecular weight of less than 1,000 Daltons.

The invention further relates to a method for preventing or treating an encephalomyelopathic or demyelinating disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons.

In addition to the therapeutic methods, the invention also provides a pharmaceutical composition, in particular for the prevention or treatment of an encephalomyelopathic, demyelinating or autoimmune disease, comprising a component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons, excluding the compound of claim 1 of WO 90/03178, effective to prevent further deterioration of a symptom of the disease and a pharmaceutically acceptable carrier. In specific embodiments, the pharmaceutical composition contains a dosage of the component of venom having a molecular weight of less than about 12,000 Daltons corresponding to an amount present in from about 5 µg/kg/day of whole venom to about 500 µg/kg/day of whole venom. Preferably, the dosage corresponds to an amount present in from about 25 µg/kg/day of whole venom to about 150 µg/kg/day of whole venom.

The invention further relates to a pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract.

The invention further relates to a pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *Pseudomyrmex triplarinus* ant.

The invention further relates to a pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons and more than about 1,000 Daltons.

The invention further relates to a pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *Pseudomyrmex triplarinus* ant, and which contains one or more components having an apparent molecular weight of less than 1,000 Daltons.

In addition, as described for the methods of the invention, the invention also provides for pharmaceutical compositions comprising other agents that modulate the activity or side effects of the low molecular weight fraction of hymenoptera venom. For example, the pharmaceutical composition can comprise an anesthetic or an analgesic. Alternatively, the pharmaceutical composition can comprise an anti-inflammatory drug, for example, a non-steroidal anti-inflammatory drug, a steroid, a non-inflammatory cytokine such as interferon-β, or an inhibitor of an inflammatory cytokine.

Furthermore, the present invention relates to use of a composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons, excluding the compound of claim 1 of WO 90/03178, for the manufacture of a medicament for the treatment of an encephalomyelopathic, demyelinating or autoimmune disease.

The invention further relates to a pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons for the prevention or treatment of an encephalomyelopathic or demyelinating disease.

The invention further relates to the use of a composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons for the manufacture of a medicament for the prevention or treatment of an encephalomyelopathic or demyelinating disease.

Finally, the present invention relates to a method of preparing a pharmaceutical composition for the treatment of an encephalomyelopathic disease comprising the step of fractionating whole hymenoptera venom into a fraction containing components having an apparent molecular weight greater than about 12,000 Daltons and a fraction containing components having an apparent molecular weight of less than about 12,000 Daltons using an ultrafiltration cell having a 10 kilo-Dalton cut-off membrane.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a plot of mean clinical scores versus time for various groups of mice treated according to the method of the invention.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is directed to a method for treating an encephalomyelopathic, demyelinating or autoimmune disease, more particularly, an autoimmune encephalomyelopathic disease, and preferably an encephalomyelopathic disease such as multiple sclerosis. According to the invention, a therapeutically effective amount of a component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is administered to a subject

suffering from the disease. Preferably the venom component is effective to prevent further deterioration of a symptom of the disease. Preferably, the hymenoptera venom is apid venom or vespid venom. In a specific embodiment, the venom is honey-bee *(Apis mellifera)* venom.

In addition to the therapeutic methods, the invention also provides a pharmaceutical composition for the treatment of an encephalomyelopathic, demyelinating or autoimmune disease comprising a component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons effective to prevent further deterioration of a symptom of the disease and a pharmaceutically acceptable carrier. In specific embodiments the pharmaceutical composition contains a dosage of the component of bee venom having a molecular weight of less than about 12,000 Daltons corresponding to an amount present in from about 5 µg/kg/day of whole bee venom to about 500 µg/kg/day of whole bee venom. Preferably, the dosage corresponds to an amount present in from about 25 µg/kg/day of whole bee venom to about 150 µg/kg/day of whole bee venom.

The invention also provides therapeutic methods and associated pharmaceutical compositions comprising other agents that modulate the activity or side effects of the low molecular weight fraction of hymenoptera venom. For example, the pharmaceutical composition can comprise an anesthetic or an analgesic. Alternatively, the pharmaceutical composition can comprise an anti-inflammatory drug, for example, a non-steroidal anti-inflammatory drug, a steroid, or a cytokine such as interferon-β.

In a preferred embodiment of the method of the invention, the method further comprises administering a component of

hymenoptera venom having a molecular weight of more than about 12,000 Daltons. It is believed that some components of the fraction of hymenoptera venom having a molecular weight of more than about 12,000 Daltons show a synergistic or enhancing therapeutic effect in treating encephalomyelopathic, demyelinating or autoimmune diseases when used in combination with the component of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons. Examples of such further components of hymenoptera venom having a molecular weight of more than about 12,000 Daltons are phospholipases.

The invention is based, in part, on the observation described in the Examples herein that a low molecular weight fraction of honey-bee venom is effective for treating a model encephalomyelopathic diseases, EAE. The effectiveness of the treatment can be seen from a decrease in the rate and extent of deterioration associated with EAE in animals treated with the low molecular weight (12,000 Dalton cut-off with an ultrafiltration membrane) as determined from behavioral criteria and histopathology.

As use herein, a "subject" includes a patient, individual, or other related term for an animal receiving treatment according to the invention. A subject is an animal for whom administration of a component of hymenoptera venom is an effective therapeutic regimen for an encephalomyelopathic, demyelinating or autoimmune disease, and is preferably a mammal, and more preferably a human, but can be any animal. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods and pharmaceutical compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, domestic animals, such as feline or

canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., *i.e.,* for veterinary medical use.

The invention provides treatment methods and compositions for a subject suffering from an encephalomyelopathic, demyelinating or autoimmune disease, *e.g.,* multiple sclerosis, canine distemper, or as demonstrated in a specific example, *infra,* EAE. As used herein, the term "subject suffering from" refers to a subject who has been diagnosed, and is therefore believed, to be suffering from the disease.

Diagnosis of an encephalomyelopathic, demyelinating or autoimmune disease is accomplished by evaluation of symptomology, clinical history, and diagnostic tests. In particular, diagnosis of multiple sclerosis can be established by evaluation of symptomology, e.g. degree of paralysis, incoordination, loss of sensation, tremor, nystagmus, blindness, disturbance of speech, and bowel and bladder incontinence. Visual evoked potentials, to detect optic neuritis as well as auditory potentials to detect lesions involving the pontine auditory pathway, can be used to establish a diagnosis of MS. Evidence of white matter plaques, detected preferably by magnetic resonance imaging, generally confirms an encephalomyelopathic disease diagnosis, particularly for MS. Criteria for a diagnosis of MS classically involve identification of two distinct neurological lesions removed in space and time. For example, a subject may present with a complaint involving visual acuity (optic neuritis), and at a later time (probably after the visual problem has resolved), with a different complaint,

perhaps relating to balance. In addition, clinical history may be important in a diagnosis of an encephalomyelopathic disease, particularly MS.

Hymenoptera Venom

The terms "a component of hymenoptera venom" and "the component of hymenoptera venom" as used herein means one or more components of hymenoptera venom.

The term "venom" as used herein refers to the product of hymenoptera venom glands containing a complex mixture of biogenic amines, amino acids, peptides, proteins, fragments of proteins, e.g. degradation products of proteins, and enzymes, which demonstrate potent pharmacological, and in some individuals, allergological effects.

As used herein, the term "hymenoptera" refers to any of the order of specialized venomous insects including bees, social wasps, ants, etc. Preferably, hymenoptera refers to insects of the families Apidae and Vespidae.

As used herein, the term "apid" is used according to the practice of those in the field and refers to insects belonging to the worldwide family of Apidae, including but not limited to bees (genus *Apis)*, bumble bees (genus *Bombus)*, carpenter bees (genus *Xylocopa)*. The preferred species of venom is from the European honeybee, *Apis mellifera* (including the European *Apis mellifera mellifera* and African *Apis mellifera adansoni)*, the Eastern honeybee *Apis cerana*, the giant honeybee *Apis dorsata* and the little honeybee *Apis florea*. Species in the genus *Bombus* include but are not limited to *B. impatiens, B. terestris, B. huntii, B. occidentalis, B. atratus, B. ignitus,* and Megabombus pennsylvanicus.

Species in the genus Xylocopa *include but are not limited to* X. violacea, X. virginica, X. appendiculata, *and* X. canescens.

As herein, the term "vespid" is used according to the practice of those in the field of allergy, and refers to insects belonging to the worldwide family of Vespidae, *i.e.*, social wasps including hornets, yellowjackets, and paper wasps. In particular, vespids include the subfamilies Vespinae and Polistinae. More particularly, the vespids include the genera *Vespa* Linnaeus, *Vespula* Thomson, *Dolichovespula* Rohwer, and *Polistes* Latreille. Species in the genus *Vespula* include but are not limited to *V. germanica* (Fab.), *V. squamosa* (Drury), *V. maculifrons* (Buysson), *V. flavopilosa* (Jacobson), *V. vulgaris* (L.), and *V. pensylvanica* (Saussure). Species in the genus *Polistes* include but are not limited to *P. annularis* (Linnaeus), *P. exclamans* (Viereck), *P. metricus* (Say), *P. fuscatus* (Fabricius), and *P. apachus* (Saussure). Species in the genus *Dolichovespula* include but are not limited to *D. maculata* (L.) and *D. arenaria* (Fab.). Species in the genus *Vespa* include but are not limited to *V. crabro* (L.) and *V. orientalis* (Linnaeus).

In connection with the present invention the component of hymenoptera venom has an apparent molecular weight of less than about 12,000 Daltons. In a specific embodiment of the present invention, the component of hymenoptera venom has an apparent molecular weight of less than about 10,000 Daltons. In a further specific embodiment of the present invention, the component of hymenoptera venom has an apparent molecular weight of less than about 5,000 Daltons.

As used herein the term "a molecular weight of less than about 12,000 Daltons" or "low molecular weight" refers to

an apparent molecular weight equal to or less than about 12,000 Daltons. The term substantially excludes an apparent molecular weight of greater than about 12,000 Daltons. However, as one of ordinary skill in the art can readily appreciate, molecular weight cut-offs are empirical, and separations are a function of molecular configuration of and conformation, especially of a macromolecule, as well as characteristics of the separation process, and are not identical to molecular mass. Thus, the closer a molecule's molecular weight is to the molecular weight cut-off, the more likely it is to be found in both fractions of a separation process. It can be readily appreciated that such cut-offs are susceptible to anomalies. Therefore, the term "less than 12,000 Daltons" refers to the distribution of venom molecules using a 12,000 Dalton cut-off. Fractionation can be effected by a number of different techniques, including but not limited to, ultrafiltration, gel filtration (size exclusion chromatography), electrophoresis (including preparative gel electrophoresis), densitometry (ultracentrifugation), and other liquid phase size fractionation techniques. Further purification can then be achieved using standard techniques, such as ion exchange chromatography, reverse phase chromatography, salting out chromatography, affinity chromatography, etc.

A "low molecular weight component" of hymenoptera venom (referred to herein alternatively as a "venom component or merely "component") is a venom component comprising a molecule or molecules having an apparent molecular weight of less than or about equal to 12,000 Daltons. Such molecular weight is an operative molecular weight: that is, it is an apparent molecular weight determined by the partitioning of a component after one or more fractionation steps. The apparent or operative molecular

weight may be greater or smaller than the actual molecular mass, as those of ordinary skill in the art can readily appreciate. Preferably, a low molecular weight component comprises a molecule or molecules having an apparent molecular weight that is within 1.5 standard deviations of the molecular weight cut-off of a given size fractionation procedure. The compositions of the invention may comprise one or more low molecular weight molecules. In a specific embodiment, the component comprises all of the lower molecular weight molecules of honey-bee venom obtained by size fractionation using ultrafiltration with a 12,000 Dalton MW cut-off.

Molecules of hymenoptera venom having "a molecular weight of less than about 12,000 Daltons" may be defined as those molecules, of which at least 10 % by number will penetrate an Amicon ultrafiltration cell, model 8050 equipped with a YM10 DIAFLO 10 kD cut-off membrane at pH 4.

Where the low molecular weight component comprises the molecules of a venom having a molecular weight of less than 12,000 Daltons, preferably two or more of these molecules are present in approximately the same proportion as found in unfractionated venom. The term "approximately" in connection with the proportion of molecules in a low molecular weight venom component means within an order of magnitude (a factor 10), preferably within a factor of five, and more preferably within a factor of two.

A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc.) when at least about 75 % by weight of the

proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, `which is substantially free of contamination, contain only a single class of molecular weight species having the activity or characteristic of the species of interest.

The claims of the present application includes a disclaimer having the following wording: "excluding the compound of claim 1 of WO 90/03178". Claim 1 of WO 90/03178 has the following wording:

"1. An anti-inflammatory and analgesic compound having an infrared absorption spectrum having characteristic absorption at about 3120 cm^{-1}, 3020 cm^{-1}, 1480 cm^{-1}, 1400 cm^{-1} and 950 cm^{-1} with no characteristic absorption at about 1640 cm^{-1} and 1540 cm^{-1}."

The disclaimer excludes the subject-matter, wherein the compound of claim 1 of WO 90/003178 alone constitutes the component of hymenoptera venom, whereas mixtures of the compound of claim 1 of WO 90/03178 and one or more (other) therapeutically effective components of hymenoptera venom are not excluded.

Low Molecular Weight Molecules of Bee Venom

The molecules that make up bee venom are summarized in Table 1, above. Bee venom components are reviewed in a number of references, including Habermann,1972, Science 177:314-322; Banks and Shipolini, 1986, In *Venoms of the Hymenoptera*, T. Piek (ed.), Academic Press: London, p.p. 330-416; Shipolini,1984, "Biochemistry of Bee Venom," in

Handbook of Natural Toxins, Vol. 2, A.T. Tu (ed.) Marcel Deklan, Inc.: New York, pp 49-85; Bousquet et al., in *Allergies aux Hymenoptures,* Institut Francais de Recherche en Allergologie: Joinvi!le-le-Pont; Piek, 1986, In *Venoms of the Hymenoptera,* T. Piek (ed.), Academic Press: London, pp. 417-424; and Mueller, 1990, in *Insect Sting Allergy. Clinical Picture, Diagnosis and Treatment,* translated by B.N. Chandler-Lorenz, Gustav Fischer: Stuttgart, pp. 12-29. The low molecular weight component molecules are discussed in more detail below.

Biogenic am in es. The presence of the most important biogenic amine, histamine, in been venom was first shown in 1936. Values of around 1 % histamine content in bee venom have been measured (Mueller, *supra)*. Bee venom also contains smaller quantities of noradrenaline and dopamine. Along with the biogenic amines, one can find free amino acids, oligopeptides, phospholipids, and carbohydrates in bee venom. Bumble bee venom may also contain small amounts of serotonin, putrescine (found in *B. ignitus),* and acetylcholine.

Peptides. Melittin is the main component of bee venom, constituting over 50% of total dry weight. This basic peptide is made up of 26 amino acids and has a molecular weight of 2840 Daltons. Melittin, because of its chemical structure, forms micelles in solution, which evidence a higher molecular weight. Melittin has a strongly basic, hydrophilic C-terminal end and a hydrophobic N-terminal end thus showing the structure of an invert soap, which not only explains its surfactant activity as a true detergent, but also its extreme membrane-toxicity on a cellular and on a subcellular level. The damage to cell membranes is chiefly brought about by an increase of permeability resulting, for example in augmentation of the potassium outflow and later in cytolysis. Its

interaction with cellular organelle membranes results in liberation of enzymes from lysosomes or of effects of melittin. Melittin reacts too with membrane-associated enzyme systems. For example, it damages the cation-activated membrane ATP-ases and acetylcholinesteerases, and interrupts oxidative phosphorylation in the mitochondria. Such cellular and subcellular effects explain the toxicity of this substance to various organ systems. Melittin induces generalized convulsions and myoclonus.

Closely related to melittin (a peptide of honey bee venom) are the bombolitins, which are potent in mast cell degranulating peptides from bumble bees (argiolas and Pistano, 1984, J. Biol. Chem. 260:1437A4).

Apamine is also a basic peptide, consisting of 18 amino acids, with a molecular weight of 2027 Daltons. It comprises about 2% of the dry weight of bee venom. Along with a strongly basic nature its high sulphur content - in common with other animal-derived neurotoxins - is notable. It causes convulsions and myoclonus. In animal experiments apamine induces hypermotility, hyperexcitability, and toniccloning cramps of long duration, due to central nervous damage. Apamine blocks Ca^{++}-dependant potassium channels. Apamine-sensitive potassium channels have been observed to participate in the mechanism that generates myotonia in myotonic dystrophy (Behrens et al., 1994, Muscle & Nerve 17: 1264-1270). Potassium channel openers have been found to enhance T cell responses to bovine peripheral myelin, and concomitantly suppress the number of gamma interferon (IFN-γ) secreting cells, in the experimental allergic neuritis disease model (Mix et al., 1994, Autoimmunity 18:233-241).

Mast cell degranulating (MCD)-peptide (Peptide 401) is closely related structurally to apamine but even more strongly basic. Consisting of 22 amino acids, with a molecular weight of 2588 Daltons, it constitutes about 1 % of the dry weight of bee venom. It is named after its extreme effect of causing mast cells to release histamine, 10 to 100 times stronger than that of melittin. This effect is not mediated through membrane toxicity. It is probably the highly basic nature of this molecule which is important for fixing it to mast cell receptors. It may also work by displacing histamine from its salt-like binding to heparin.

Secapine is a 25 amino acid peptide comprising 0.5% of the dry weight of venom. It is not very toxic and its significance is unknown.

Tertiapine is a basic peptide composed of 21 amino acids, structurally similar to the mast cell degranulating peptide and apamine. It comprises 0.1 % of the dry weight of bee venom. Like MCD-Peptide it causes mast cells to degranulate, but is much less potent.

Cardiopep is a poorly characterized, low molecular weight peptide, with positive chronotropic and positive inotropic actions on the heart.

Small Proteins. A protease inhibitor with a molecular weight of around 9000 Daltons has been found in bee venom. In addition, a basic polypeptide with a molecular weight of 11092, called adolapin because of its analgetic action, has been found. *In vitro,* adolapin blocks the metabolism of arachidonic acid by inhibiting cyclo-oxygenase and lipoxygenase. In addition, small amounts of phospholipase A_2, which has a molecular weight of 19,000

Daltons, may be included in the low molecular weight component of bee venom.

Low Molecular Weight Molecules of Vespid Venom

The molecules of vespid venom are summarized in Table 2, above. The low molecular weight component molecules are discussed in more detail below.

Biogenic amines. The histamine content of vespid venom is significantly higher than that of honey bee venom, being around 4% for *Vespula,* 3% for *Polistes,* and 5-6% for *Dolichovespula.* Venoms of all Vespidae also contain serotonin, and hornet venom acetylcholine. Dopamine, noradrenaline and adrenaline have also been found in vespid venoms.

Peptides and small proteins. The venoms of all Vespidae contain kinins, which are lower molecular weight peptides with 10-20 amino acids, sometimes with carbohydrate moieties. Kinins lead to smooth muscle contractions, blood pressure drop and increased tissue permeability. A peptide with mast cell degranulating activity has been reported in vespid venom. A peptide similar to melittin, with a molecular weight of 6000 Daltons and with direct hemolytic activity has been identified in vespid venom as well. These peptide are likely to be responsible for most of the toxic effects of vespid venoms.

Furthermore, the venoms of Vespidae contain mastoparans and Antigen 5, and *Vespa mandarinia* specifically contains mandarotoxin. Mastoparans are peptides from Vespid venoms that stimulate the release of histamine and stimulate phopholipase activity (Argiolas and Pisano, J. Biological Chem., v258, p13697-13702, 1983). Although their actions are similar, there is no sequence homology between the

vespid mastoparans and the MCD and melittin peptides of honey bee venom. Vespid venom Antigen 5 and specifically mandarotoxin from *Vespa mandarinia* do not exhibit any known enzyme activities, but there is some evidence of neurotoxic activity (Nakajima T, IN: "Venoms of the Hymenoptera", Piek T, editor, Academic press, 1986, p 309-327).

Source of Venom Components

In addition to isolation of a low molecular weight component of hymenoptera venom from natural venom, the present invention contemplates that an individual molecule or molecules can be obtained from other sources. For example, the biogenic amines can be obtained by chemical synthesis, or purchased from commercial sources. The peptides and small proteins can be obtained synthetical!y, *e.g.*, by the well-known methods of solid phase peptide synthesis, or recombinantly, e.g., using the methods as described in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. G!over ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* `(1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therapy

As noted above, the present invention provides administration of a low molecular weight component (including a low molecular weight fraction of whole venom) of a hymenoptera venom to a subject for the treatment of an encephalomyelopathic, demyelinating or autoimmune disease. In a specific embodiment, *infra*, administration of a low molecular weight fraction (less than about 12,000 Daltons) of honey-bee venom is effective in reducing symptoms of EAE in mice, which is a model for multiple sclerosis.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 5 percent, preferably by at least 15 percent, more preferably by at least 50 percent, still more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function, and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host. In specific embodiments, a therapeutically effective amount will slow degeneration, preferably prevent further degeneration, and more preferably improve, a symptom of encephalomyelopathic disease, such as, but not limited to, paralysis, incoordination, loss of sensation, tremor, nystagmus, blindness, disturbance of speech, and bowel and bladder incontinence. In another embodiment, a therapeutically effective amount will slow the rate of deterioration of vision, preferably prevent further deterioration of vision, and more preferably improve vision, *e.g.*, as measured by visual evoked potentials to detect optic neuritis. In yet a further embodiment, a therapeutically effective amount will reduce the rate of formation of, preferably inhibit formation of, and more preferably reverse the number of,

white matter plaques, *e.g.,* as detected by magnetic resonance imaging (MRI).

According to the invention, the component or components of a therapeutic composition of the invention may be introduced parenterally, transmucosally, *e.g.,* orally, nasally, or rectally, or transdermally. Preferably, administration is parenteral, *e.g.,* via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

In another embodiment, the component of the therapeutic composition can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer,* Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally *ibid).*

In yet another embodiment, the component of the therapeutic composition can be delivered in a controlled release system. For example, the component may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release,* Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance,* Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev.

Macromol. Chem. 23:61(1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351(1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain and pheripheral neurons, thus requiring only a fraction of the systemic dose *(see, e.g.,* Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Preferably, a controlled release device is introduced into a subject in proximity of the site of inappropriate immune activation or a tumor.

Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), *i.e.* the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects.

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

Because demyelination is a continuous process, controlled release administration is desirable.

As noted above, the venom component of the invention can be isolated from hymenoptera venom, synthesized, or produced recombinantly.

In a further aspect, recombinant cells that have been transformed with one or more of the effective low molecular weight component or components of hymenoptera venom and that express high levels of the component(s) can be transplanted in a subject. Preferably autologous cells transformed with a gene encoding such a component or components are transplanted to avoid rejection;

alternatively, technology is available to shield non-autologous cells that produce soluble factors within a polymer matrix that prevents immune recognition and rejection.

Thus, the low molecular weight component can be delivered by intravenous, intraarterial, intraperitoneal, intramuscular, or subcutaneous routes of administration. Alternatively, the low molecular weight component of hymenoptera venom, properly formulated, can be administered by nasal or oral administration. A constant supply can be ensured by providing a therapeutically effective dose *(i. e.,* a dose effective to modulate a symptom or manifestation of an encephalomyelopathic disease in a subject) at the necessary intervals, *e.g.,* daily, every 12 hours, etc. These parameters will depend on the severity of the disease condition being treated, other actions, such as diet modification, that-are implemented, the weight, age, and sex of the subject, and other criteria, which can be readily determined according to standard good medical practice by those of skill in the art.

<u>Derivatives of a Low Molecular Weight Component of Hymenoptera Venom</u>

Generally, one or more molecules present in the low molecular weight component may be derivatized by the attachment of one or more chemical moieties to the molecule. The chemically modified derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular, subcutaneous, intravenous, oral, nasal, pulmonary, topical, or other routes of administration. Chemical modification of a biologically active molecule may provide additional advantages under certain circumstances, such as increasing the stability and

circulation time of the molecule and decreasing immunogenicity. *See* U.S. Patent No. 4,179,337, Davis et al., issued December 18, 1979. For a review, *see* Abuchowski et al., in *Enzymes as Drugs* (J.S. Ho!cerberg and J. Roberts, eds. pp. 367-383 (1981)). A review article describing protein modification and fusion proteins is Francis, 1992~ *Focus on Growth Factors 3:4-10,* Mediscript: Mountview Court, Friern Barnet Lane, London N20, OLD, UK.

Chemical Moieties For Derivatization. The chemical moieties suitable for derivatization may be selected from among water soluble polymers. The polymer selected should be water soluble so that the molecule to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/component conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present venom component, these may be ascertained using the assays provided herein. The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol; carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, póly-1 ,3, 6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol.

Polyethylene glycol propionaldenhyde may advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene g!ycol, the preferred molecular weight is between about 2kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile *(e.g.,* the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties *(e.g.,* polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to component or components molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted component or components and polymer) will be determined by factors such as the desired degree of derivatization *(e.g.,* mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the molecule with

consideration of effects on the molecule's functional domains. There are a number of attachment methods available to those skilled in the art, *e.g.,* EP 0 401 384 herein incorporated by reference (coupling PEG to G-CSF), *see also* Malik et al., 1992, Exp. Hematol. 20:1028-1035 (reporting pegy!ation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group include lysine residues and the N- terminal amino acid residues; those having a free carboxyl group include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

Furthermore, the derivatives of the low molecular weight component of hymenoptera venom suitable for use in the present invention include fragments of the said component, in particular fragments of peptides. Such smaller fragments possess the advantage that they retain therapeutic activity while minimizing or eliminating detrimental effects.

Pharmaceutical Compositions

In yet another aspect of the present invention, provided are pharmaceutical compositions of the above. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal, or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising

effective amounts of a low molecular weight venom component, including a lower molecular weight venom component comprising a derivitized molecule, of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content *(e.g.,* Tris-HCI, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents *(e.g.,* Tween 80, Polysorbate 80), anti-oxidants *(e.g.,* ascorbic acid, sodium metabisulfite), preservatives *(e.g.,* Thimersol, benzyl alcohol) and bulking substances *(e.g.,* lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in* vivorelease, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.,* Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. In a specific, preferred embodiment of the invention, the term "pharmaceutically acceptable" as used herein means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The

term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

Oral Delivery. Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers *(e.g.,* U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the venom component and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above derivatized molecules of the venom component. The molecules may be chemically modified so that oral

delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the molecule itself, where said moiety permits (a) inhibition of hydrolysis, and/or (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the active molecule present in the component, increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Abuchowski and Davis, 1981, "Soluble Polymer-Enzyme Adducts" In: *Enzymes as Drugs,* Hocenberg and Roberts, eds., Wiley-Interscience, New York, NY, pp. 367-383; Newmark, et al., 1982, J. Appl. Biochem. 4:185-189. Other polymers that could be used are poly-1 ,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

For the component (or derivative) the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the molecule (or derivative) or by release of the biologically active material beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT),

hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic *i.e.*, powder; for liquid forms, a soft gelatin shell may be used The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, a-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans, and starch. Certain inorganic salts may be also be used as

fillers including calcium triphosphate, magnesium carbonate, and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress, and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include but are not limited to starch, including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge, and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An anti-frictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin,

vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica, and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative dither alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the molecule (or derivative) are for instance the fatty acids oleic acid, linoleic acid, and linolenic acid.

Controlled release oral formulation may be desirable. The drug could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms, *e.g.*, gums. Slowly degenerating matrices may

also be incorporated into the formulation. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating paid. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the non-enteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid. A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Pulmonary Delivery. Also contemplated herein is pulmonary delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. Other reports of this include Adjei et al., 1990, Pharmaceutical Research, 7:565-569; Adjei et al., 1990, International Journal of Pharmaceutics, 63: 135-144 (leuprolide acetate); Braquet et al., 1989, Journal of Cardiovascular Pharmacology, 13 (suppl. 5): 143-146 (endothelin-1); Hubbard et al., 1989, Annals of Internal Medicine, Vol. III, pp. 206-212 (α1-antitrypsin); Smith et al., 1989, J. Clin. Invest. 84:1145-1146 (α-1-proteinase); Oswein et al., 1990, "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colorado, March, (recombinant human growth hormone); Debs et al., 1988, J.

Immunol. 140:3482-3488 (interferon-γ and tumor necrosis factor alpha) and Platz et al., U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor).

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

- All such devices require the use of formulations suitable for the dispensing of protein (or derivative). Typically, each formulation is specific to the -type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified protein may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise protein (or derivative) dissolved in water at a concentration of

about 0. 1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar *(e.g.,* for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the venom component suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1, 1, 1 ,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing protein (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, *e.g.,* 50 to 90% by weight of the formulation. The protein (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 mm (or microns), most preferably 0.5 to 5 mm, for most effective delivery to the distal lung.

Nasal Delivery. Nasal delivery of the protein (or derivative) is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose,

without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

The term "mucosal penetration enhancer" refers to a reagent that increases the rate or facility of transmucosal penetration of a venom component, particularly for nasal administration, such as but not limited to, a bile salt, fatty acid, surfactant or alcohol. In specific embodiments, the permeation enhancer can be sodium cholate, sodium dodecyl sulphate, sodium deoxycholate, taurodeoxycholate, sodium glycocholate, dimethylsulfoxide or ethanol. Suitable penetration enhancers also include glycyrrhetinic acid (U.S. Patent No. 5,112,804 to Kowarski) and polysorbate-80, the latter preferably in combination with an non-ionic surfactant such as nonoxynol-9, laureth-9, poloxamer-124, octoxynol-9, or lauramide-DEA (European Patent EP 0 242 643 B1 by Stoltz).

Methods of Treatment Methods of Preparing a Medicament

In yet another aspect of the present invention, methods of treatment and manufacture of a medicament are provided. Conditions alleviated or modulated by the administration of the present derivatives are those indicated above and in greater detail below.

Dosages. For all of the above components, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of encephalomyelopathic disease conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain proper dosing. As shown in the Examples, *infra,* a dosage approximately

corresponding to the equivalent of 5 μg/kg/day of whole venom to about 500 μg/kg/day of whole venom, and more preferably, from about 25 μg/kg/day to about 150 μg/kg/day of whole venom, is therapeutically effective in the EAE disease model. Generally, for intravenous injection or infusion, or with a derivatized molecule of the component, dosage may be lower. The dosing schedule may vary, depending on the circulation half-life, and the formulation used.

Administration with other compounds. For treatment of an encephalomyelopathic disease, one may administer the present component or components with another agent or compound, such as an anesthetic or analgesic. Examples of anesthetics include, but are not limited to, lidocaine, procaine, ketamine, ... Examples of analgesics include, but are not limited to non-steroidal anti-inflammatory agents (salicylic acid, acetaminophen, ibuprofin), narcotics (morphine, fentanyl, etc.), ...

In another embodiment, an anti-inflammatory drug is administered in conjunction with the low molecular weight component or components of hymenoptera venom. Examples of anti-inflammatory drugs include, but are not limited to, non-steroidal anti-inflammatory drugs, steroids *(e.g.,* cortisone), immunosuppressive drugs *(e.g.,* cyclosporin), and inflammation suppressive cytokines (interleukin-6, interferon-β), and antagonists of inflammatory molecules (anti-IL-1 antibody, anti-TNF-antibody).

Administration of the venom component and the additional agent may be simultaneous (for example, administration of a mixture of the present component and IFN-β) or may be *in seriatim*. Thus, the agent can be administered at the same time as, prior to, or after administration of the venom component, provided - that the agent is

pharmacologically active at the time of administration of the venom component.

Encephalomyelopathic, demyelinating and autoimmune diseases

As noted above, the present invention is directed to the treatment of an encephalomyelopathic, demyelinating or autoimmune disease. The said three groups of diseases are to a large extent overlapping as will appear from the account given in the following.

Examples of encephalomyelopathic diseases include, but are not limited to, multiple sclerosis (MS); disseminated sclerosis; focal sclerosis; insular sclerosis; tabes dorsalis (posterior sclerosis); acute and chronic experimental allergic (or autoimmune) encephalomyelitis (EAE), an animal model of MS; Guillain-Barré syndrome; experimental allergic neuritis (an animal model of Guillain-Barré syndrome); acute disseminated encephalomyelitis; myalgic encephalomyelitis (benign and epidemic); viral encephalomyelitis; granulomatous encephalomyelitis; etc. Also included are animal diseases, such as but not limited to canine distemper; feline distemper; equine encephalomyelitis (eastern, Venezuelan, and western); avian encephalomyelitis; porcine encephalomyelitis; bovine encephalomyelitis; mouse encephalomyelitis; etc.

Examples of demyelinating diseases include, but are not limited to, multiple sclerosis (MS), disseminated sclerosis (DS), acute disseminated encephalomyelitis, progressive multifocal leukoencephalopati (PML), and subacute sclerotic panencephalitis (SSPE).

In connection with the present invention, the term "autoimmune diseases" include, but is not limited to, mutiple sclerosis (MS), rheumatoid arthritis (RA), insulin-dependant diabetes mellitus (IDDM), hyperthyroidism (Graves disease), myasthenia gravis (MG), systemic lupus erythematosus (SLE), and polyartheritis nodosa. A noticeable characteristic of autoimmune diseases is their familial clustering and association with the expression of particular genes, in particular genes of class I and class II major histocompatibility complex (MHC). For example, a large proportion of MS patients have the HLA-DR2 haplotype (Beall SS, Concannon P, Charmley P, et al.,J. Neuroimmunol.,v 21, p59-66, 1989). Since not all subjects with a susceptible genotype develop the autoimmune disease, it appears that environmental factors also play a major role. For example, MS appears to be more common in subjects who live in temperate climate regions (Kurtzke JF, IN: Multiple Sclerosis, Hallpike JF, Adams CWM, Tourtelotte WW, editors, Williams and Wilkins, Baltimore, MD, 1983, P 49-95). It has long been speculated that the environmental factor of autoimmune diseases may be an infectious agent such as a virus. The etilogy of several human and animal diseases can be attributed to viral infection. For example, Theiler's murine encephalomyelitis is a demyelinating disease with clinical and pathological signs similar to EAE. Although antibodies are involved in some effector responses of autoimmune diseases, the triggering event in most cases begins with the activation of CD4 T-cells that are required for B cell maturation and clonal expansion.

In particular, the present invention is directed to the treatment of neurological sclerotic diseases with an inflammatory or autoimmune component. Thus, in a preferred aspect, the present invention is directed to

treatment of an autoimmune encephalomyelopathic condition.

In the most preferred embodiment, the invention is directed to treatment of multiple sclerosis.

The present invention will be better understood by reference to the following non-limiting examples, which are provided by way of exemplification.

EXAMPLE 1

A LOW MOLECULAR WEIGHT FRACTION OF BEE VENOM REDUCES PATHOGENESIS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS.

To demonstrate the therapeutic effects of different *Apis mellifera* honey bee venom (HBV) preparations on the onset and development of demyelinating disease, the acute experimental allergic encephalomyelitis (EAE) mouse model was used. After the induction of EAE in mice, HBV treatments were initiated, and the extent of disease and its progression were observed by measuring changes in physical activity and mobility associated with EAE. The physical disabilities associated with onset and progression of disease were assessed through both clinical and histological means described herein. This Example demonstrates that a low molecular weight fraction of HBV reduces pathogenesis associated with EAE as determined by clinical and histopathological criteria. The Examples are divided into bee venom fractionation and administration of fractionated bee venom to mice in an EAE model.

FRACTIONATION OF HONEY BEE VENOM

Materials and Methods

Fractionation of HBV. Amicon ultrafiltration cell, model 8050 equipped with a YM10 DIAFLO 10kD cut-off membrane, was used in the fractionation of raw *Apis mellifera* venom. One mg/ml raw *Apis mellifera* venom in 0.1 M ammonium acetate, pH 4 was filtered through a 45 µM filter (Millipore Millex-HV). A 50 ml solution at 5°C was added to the filtration container; stirring and pressure (4.0 bar; nitrogen gas) were applied. Diafiltration was continued until approximately 5 ml of solution was left in the container, the pressure was relieved, and the container was refilled to 50 ml using 0.1 M ammonium acetate, pH 4.0. One hundred µl samples were taken from the filtrate and retentate and the entire procedure was repeated 10 times to obtain a high molecular weight fraction (>10 kD) and a low molecular weight fraction (<10 kD). Samples taken before, during and after filtration were analysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The retentate and 10 pooled filtrates were lyophilized. The lyophilized fractions were reconstituted in 5 ml of 5 mM ammonium acetate, pH 4 and analysed by SDS-PAGE using standards with known molecular weight. Three individual batches were prepared according to the procedure described and fractions from each batch compared by SDS-PAGE.

The SDS-PAGE analysis showed that the separation of molecules having molecular weights of less than 10 kD and more than 10 kD, respectively, was substantially complete, whereas both fractions showed a band of molecules having a molecular weight of about 10 kD hence indicating only a partial separation. Furthermore, the SDS-PAGE analysis showed that the fractions from the three batches were comparable in respect to composition.

Subsequently, the filtrates from the three batches were pooled to form the low molecular weight fraction used in therapeutic effect tests described below,

EFFECT OF FRACTIONATED HBV ON EAE PATHOLOGY

Materials and Methods

Induction of Acute EAE in mice. SJL/J x BALB/c Fl mice 6 to 12 weeks of age were induced with acute EAE by inoculation with mouse spinal cord homogenate (MSCH) or mouse myelin basic protein (MBP). MSCH inoculation is performed by first emulsifying 10 mg of MSCH in complete Freund's adjuvant (CFA) with 0.4 mg of *Mycobacterium tuberculosis* H37RA (Difco) in a volume of 0.1 ml, followed by delivery of 0.05 ml of the preparation subcutaneously *(s. c.)* in each hind footpad. In addition, on the day of inoculation and 48 hours later, intravenous injections of Pertussis vaccine (2.7 x 10^{10} organisms) (Michigan Department of Public Health Laboratories) were administered.

Treatment Protocol for Acute EAE. The low molecular weight fraction obtained as described above (HBV preparation) comprising a dosage range of 0.1x, 0.5x, 1x, and 2x, where the 1x dose is calculated to contain the amount of low molecular weight protein(s) present in whole venom equal to 4 µg HBV/treatment/mouse were administered to test the effects on acute EAE. The administration of each HBV preparation was performed in 4 stages, thus in each stage a single HBV preparation having a dosage concentration of one of the four dosages described above was administered to a single group of mice. A control group was also utilized at each stage and consisted of ten mice receiving a placebo that is

indistinguishable from the HBV preparations. Starting on day 0, which marks the time of inoculation for stimulation of acute EAE, random groups of 10 mice (6 weeks of age) were injected *s.c.* with 0.1 ml of a HBV preparation (or placebo) in PBS buffer (0.01 M, pH 7.2, 0.145 M NaCl) 3 times per week until the animals were sacrificed 21 days after inoculation. Five additional mice were acquired for each stage for prestudy blood samples prior to inoculation. A blood sample was taken by cardiac puncture, and two blood smears were prepared immediately after collecting the sample. Serum was also separated and stored frozen (-20°C) for later analyses. One blood smear was stained with Wright's stain for examination of erythrocytes, leukocytes, and a differential leukocyte count. The second smear was stored for later use.

Efforts were made to keep the composition of the mouse diet consistent throughout the day, paying close attention to the intake of protein, carbohydrates, and vitamin supplements, in particular the B vitamins. The animals had *ad libitum* access to food and water, however if signs of decreased mobility appeared, adjustments were made to insure free access to food and water. Samples of food and water were also taken for chemical and microbial analyses. In addition, environmental conditions, including crowding, temperature, and relative humidity, were monitored to minimize stress to the mice and to ensure *ad libitum* access to food and water.

Assessment of EAE Pathogenesis. The pathogenesis of EAE is characterised by striking physical disabilities as a result of muscle weakness and paralysis. These physical disabilities associated with EAE can be assessed by both clinical and histological signs.

To clinically assess the condition of the mice, they were observed daily for changes in physical activity and mobility that may be attributed to the development of EAE. Generally, in the acute EAE model clinical signs begin around day 11 after inoculation and peak about day 15. These disabilities associated with EAE were scored by experienced technicians on a 10 point scale described in Table 3.

Table 3. Clinical Signs of EAE
Scoring of Physical Disabilities

0.	Asymptomatic
1.	Questionable weakness
2.	Minimal hind limb paresis and/or tail flaccidity
3.	Ataxia
4.	Minimal righting difficulty
5.	Mild hind limb paresis, difficulty righting, and tail flaccidity
6.	Moderate hind limb paresis
7.	Moderate hind limb paresis and urinary incontinence
8.	Severe hind limb paresis and incontinence
9.	Quadriparesis and incontinence
10.	Death

To histologically assess the condition of the mice after periods following inoculation, the animals were sacrificed upon reaching a clinical assessment score of 9, or 21 days after inoculation. A blood sample was taken via cardiac puncture, and two blood smears were prepared immediately after collecting the blood sample. Serum was separated and stored frozen (-20°C) for later analyses. One blood smear was stained using Wright's stain for

examination of erythrocytes, leukocytes, and a differential leukocyte count. The second smear was stored for later use.

After taking the blood sample, the animals were perfused with a 1:4 mixture of glacial acetic acid and 95% ethanol. Brain and spinal cord specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H+E stain). Then, accumulation and infiltration of inflammatory cells in the meningeal, perivascular, and parenchyma of the central nervous system were scored as indicated in Table 4.

Table 4. Pathological Signs of EAE
Scoring of Histological Specimens

0.	Normal tissue
1.	Light submeningeal infiltration
2.	Mild perivascular infiltration
3.	Marked perivascular infiltration
4.	Marked perivascular cuffing and infiltration into the surrounding parenchyma

Results

Low Molecular weight fraction.

The therapeutic effects of the low molecular weight fraction were assessed partly by mean clinical score (cf. Table 3 above) and partly by the proportion of mice still alive on day 21. The plot of the mean clinical scores by time indicates that the disease progressed in a roughly linear fashion from day 11 through day 15 (see Figure 1). The proportion of mice in each dosage group still living on day 21 and the mean clinical score on day 21 are summarized in Table 5.

Table 5.
Mean Clinical Score and Proportion Alive on Day 21

Dose	Mean Clinical Score	Proportion Alive
2x	7.3	0.5
1x	4.9	0.7
0.5x	5.6	0.8
0.1x	5.3	0.7
Placebo	6.1	0.7

As will appear from Table 5, the low molecular weight fraction used for treatment appears to inflict an adverse effect at a high dose and a favourable effect at low and medium doses. The said adverse effect is consistent with the fact that hymenoptera venom generally is believed to contain harmful constituents, possibly neurotoxins. However, the present experimental study has shown that a low molecular fraction of apid venom according to the invention at some dose levels possess therapeutic effects in an EAE model.

EXAMPLE 2

FRACTIONATION OF HONEY BEE VENOM

Materials and methods.

Fractionation of HBV. Amicon ultrafiltration cell, model 8050 equipped with a YM10 DIAFLO 10kD cut-off membrane, was used in the fractionation of raw *Apis mellifera* venom. 200 mg of raw *Apis mellifera* (Vespa Labs. Lot # HBVGAB 1941) was dissolved in 200 ml of 0.1 M ammonium

acetate, pH 4 to form a solution with a concentration of 1 mg/ml. The solution was transferred to a 400 ml Amicon Stirred Ultrafiltration Cell equipped with a 10 kDa cut-off Diaflo ultrafiltration membrane (YM10) and nitrogen pressure was applied at 4°C until 20 ml solution was retained in the cell. The flow-through (180 ml) was collected and new buffer (0.1 M NH_4Ac, pH 4.0) was added to the reservoir to a final volume of 200 ml. This procedure was repeated 10 times.

The filtrate fraction from each filtration round were individually collected and analysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE analysis showed that the filtrates of the first three filtration rounds contained mellitin in high quantities, and that no mellitin was present in the retentate after the 5^{th} filtration round. The filtrates of the first 3 filtration rounds were pooled, lyophilized and dissolved in 0.005 M NH_4Ac, pH 4.0 to give a final volume of 10 ml, and the resulting solution was used as the low molecular weight fraction in the therapeutic effect tests described below.

Quantitative amino acid analysis. The low molecular weight fraction obtained as described above as well as the 1 mg/ml raw venom solution were subjected to quantitative amino acid analysis following acid hydrolysis. The results appears from Table 6 below. The said raw venom solution had a protein/solids ratio of 0.48, whereas the low molecular weight fraction had a protein/solids ratio of 0.375 (based on a defined concentration of solids of 20 mg/ml)

Table 6. Quantitative amino acid analysis of HBV

Amino acid	Low molecular weight fraction [mol-%]	Raw venom [mol-%]
Asp	1.3	5.5
Thr	7.3	7.5
Ser	3.1	3.9
Glu	8.9	5.1
Pro	5.0	4.5
Gly	10.8	10.5
Ala	8.2	7.2
TPCys	2.4	1.6
Val	7.1	7.1
Met	ND	0.4
Ile	10.7	9.8
Leu	14.2	13.6
Tyr	ND	0.9
Phe	0.1	0.7
His	1.2	2.0
Trp	ND	ND
Lys	11.6	11.6
Arg	8.3	8.2

ND: No Data.

EFFECT OF FRACTIONATED HBV ON EAE PATHOLOGY

The induction of acute EAE in mice was carried out as in Example 1, and also the treatment protocol for acute EAE was the same as in Example 1 with the exception that each treatment group only consisted of 3 mice and that the first placebo/HBV *sc* injections were delayed until day 5. The EAE pathogenesis were assessed by mean pathology score (cf. Table 4 in Example 1).

Results.

The mean pathology scores for brain and spinal cord specimens on day 21 are given in Table 7.

Table 7.
Mean Pathology Scores on day 21

Dose	Mean Pathology Score	
	Brain	Spinal Cord
2x	2.67	2.33
1x	0.33	0.00
0.5x	0.00	0.00
0.1x	0.33	1.50
Placebo	0.67	0.33

As will appear from Table 7, the low molecular weight fraction used for treatment appears to inflict an adverse effect at a high dose and a favourable effect at low and medium doses. The said adverse effect is consistent with the fact that hymenoptera venom generally is believed to contain harmful constituents, possibly neurotoxins. However, the present experimental study has shown that a low molecular fraction of apid venom according to the invention at some dose levels possess therapeutic effects in an EAE model.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons excluding the compound of claim 1 of WO 90/03178.

2. The method according to claim 1 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is effective to reverse a symptom of the said disease.

3. The method according to claim 1 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons comprises all of the components of hymenoptera venom having a molecular weight of less than about 12,000 Daltons, which components are present in approximately the same proportion as found in natural hymenoptera venom.

4. The method according to claim 1 wherein the component of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons is prepared by fractionating whole hymenoptera venom into a fraction containing components having an apparent molecular weight greater than about 12,000 Daltons and a fraction containing components having an apparent molecular weight of less than about 12,000 Daltons using an ultrafiltration cell having a 10 kilo-Dalton cut-off membrane.

5. The method according to claim 1, wherein the hymenoptera venom is apid venom.

6. The method according to claim 5 wherein the hymenoptera venom is *Apis mellifera* venom.

7. The method according to claim 1 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is administered parenterally.

8. The method according to claim 7 wherein the parenteral administration is selected from the group consisting of intravenous, subcutaneous, intradermal, intraperitoneal, intramuscular, intraarterial, and intraventricular.

9. The method according to claim 1 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Dalton is administered orally.

10. The method according to claim 1 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Dalton is administered transmucosally.

11. The method according to claim 10 wherein the transmucosal administration is selected from the group consisting of nasal, sublingual, transbuccal, vaginal, and rectal administration.

12. The method according to claim 1 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Dalton is administered transdermally.

13. The method according to claim 1 wherein the amount of the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons corresponds to the amount present in from about 5 μg/kg/day of whole

hymenoptera venom to about 500 μg/kg/day of whole hymenoptera venom.

14. The method according to claim 13 wherein the amount of the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons corresponds to the amount present in from about 25 μg/kg/day of whole hymenoptera venom to about 150 μg/kg/day of whole hymenoptera venom.

15. The method according to claim 1 wherein the subject is a human and the said disease is multiple sclerosis.

16. The method according to claim 1 further comprising administering an anesthetic or an analgesic contemporaneously.

17. The method according to claim 16 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is administered by a route selected from the group consisting of oral, transmucosal, subcutaneous, intradermal, intraperitoneal, and intramuscular.

18. The method according to claim 1 further comprising administering an anti-inflammatory drug.

19. The method according to claim 18 wherein the anti-inflammatory drug is administered in seriatim with the component of hymenoptera venom.

20. The method according to claim 18 wherein the anti-inflammatory drug is administered simultaneously with the component of hymenoptera venom.

21. The method according to claim 18 wherein the anti-inflammatory drug is selected from the group consisting of a non-steroidal anti-inflammatory drug, a steroid, an immuno-suppressive drug, and a lymphokine/cytokine/monokine.

22. The method according to claim 21 wherein the lymphokine/cytokine/monokine is selected from the group consisting of interferon-α and interferon-β.

23. The method according to claim 1 further comprises administering a component of hymenoptera venom having a molecular weight of more than about 12,000 Daltons.

24. The method according to claim 1, wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons has a positive response in a Experimental Allergic Encephalomyelitis (EAE) model.

25. The method according to claim 1, wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is capable of blocking T-cell recognition of a myelin-associated antigen.

26. The method according to claim 1, wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is capable of inhibiting or reversing symptoms associated with an encephalomyelopathic, demyelinating or autoimmune disease.

27. A method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more

components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *pseudomyrmex triplarinus* ant.

28. A method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons and more than about 1,000 Daltons.

29. A method for preventing or treating an encephalomyelopathic, demyelinating or autoimmune disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *pseudomyrmex triplarinus* ant, and which contains one or more components having an apparent molecular weight of less than 1,000 Daltons.

30. A method for preventing or treating an encephalomyelopathic or demyelinating disease comprising administering to a subject believed to be suffering from the said disease a therapeutically effective amount of a composition comprising one or more components of the fraction of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons.

31. A pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom

having a molecular weight of less than about 12,000 Daltons excluding the compound of claim 1 of WO 90/03178.

32. The pharmaceutical composition of claim 31 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons is effective to reverse a symptom of a encephalomyelopathic, demyelinating or autoimmune disease.

33. The pharmaceutical composition of claim 31 wherein the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons comprises all of the components of hymenoptera venom having a molecular weight of less than about 12,000 Daltons, which components are present in approximately the same proportion as found in natural hymenoptera venom.

34. The pharmaceutical composition of claim 31 wherein the component of hymenoptera venom having an apparent molecular weight of less than about 12,000 Daltons is prepared by fractionating whole hymenoptera venom into a fraction containing components having an apparent molecular weight greater than about 12,000 Daltons and a fraction containing components having an apparent molecular weight of less than about 12,000 Daltons using an ultrafiltration cell having a 10 kilo-Dalton cut-off membrane.

35. The pharmaceutical composition of claim 31 wherein the hymenoptera venom is apid venom.

36. The pharmaceutical composition of claim 31 wherein the hymenoptera venom is *Apis mellifera* venom.

37. The pharmaceutical composition of claim 31 wherein the dosage of the component of hymenoptera venom having a

molecular weight of less than about 12,000 Daltons corresponds to an amount present in from about 5 µg/kg/day of whole hymenoptera venom to about 500 µg/kg/day of whole hymenoptera venom.

38. The pharmaceutical composition of claim 31 wherein the dosage of the component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons corresponds to an amount present in from about 25 µg/kg/day of whole hymenoptera venom to about 150 µg/kg/day of whole hymenoptera venom.

39. The pharmaceutical composition of claim 31 wherein the subject is a human and the said isease is multiple sclerosis.

40. The pharmaceutical composition of claim 31 further comprising an anesthetic or an analgesic.

41. The pharmaceutical composition of claim 31 further comprising an anti-inflammatory drug.

42. The pharmaceutical composition of claim 31 wherein the anti-inflammatory drug is selected from the group consisting of a non-steroidal anti-inflammatory drug, a steroid, and a lymphokine/cytokine/monokine.

43. The pharmaceutical composition of claim 42 wherein the lymphokine/cytokine/monokine is selected from the group consisting of interferon and interferon-β.

44. A pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *Pseudomyrmex triplarinus* ant.

45. A pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons and more than about 1,000 Daltons.

46. A pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding compositions, which are derived from ant venom extract from a *Pseudomyrmex triplarinus* ant, and which contains one or more components having an apparent molecular weight of less than 1,000 Daltons.

47. A method of preparing a pharmaceutical composition for the prevention or treatment of an encephalomyelopathic, demyelinating or autoimmune disease comprising the step of fractionating whole hymenoptera venom into a fraction containing components having an apparent molecular weight greater than about 12,000 Daltons and a fraction containing components having an apparent molecular weight of less than about 12,000 Daltons using an ultrafiltration cell having a 10 kilo-Dalton cut-off membrane.

48. A pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons excluding the compound of claim 1 of WO 90/03178, for the prevention or treatment of an encephalomyelopathic, demyelinating or autoimmune disease.

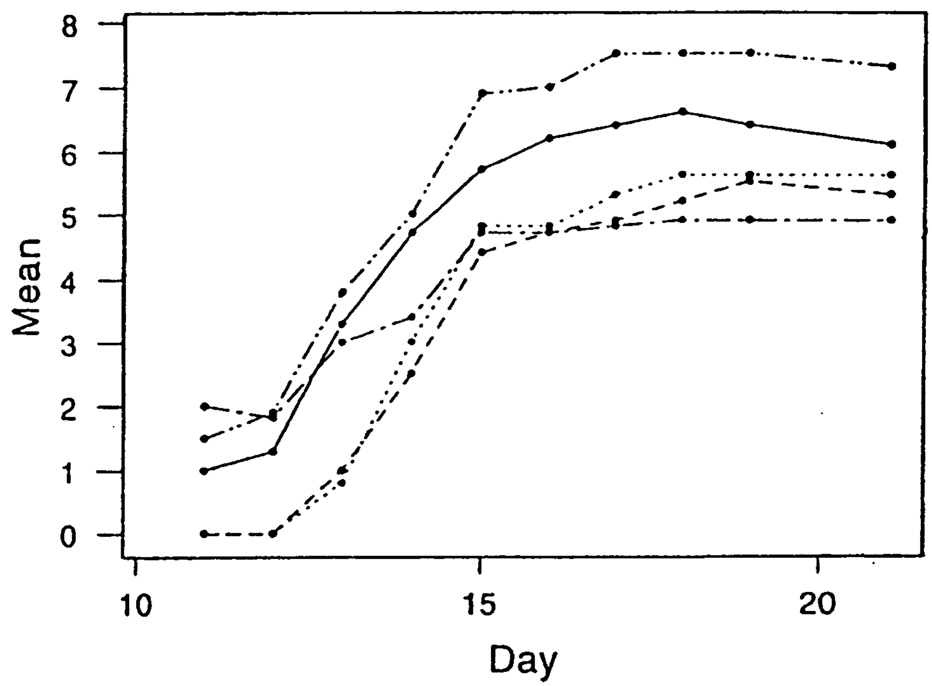
49. Use of a composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons

excluding the compound of claim 1 of WO 90/03178, for the manufacture of a medicament for the prevention or treatment of an encephalomyelopathic, demyelinating or autoimmune disease.

50. A pharmaceutical composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons for the prevention or treatment of an encephalomyelopathic or demyelinating disease.

51. Use of a composition comprising a therapeutically effective component of hymenoptera venom having a molecular weight of less than about 12,000 Daltons for the manufacture of a medicament for the prevention or treatment of an encephalomyelopathic or demyelinating disease.

Mean Clinical Score



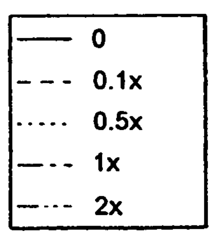


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00448

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 35/64
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS, EMBASE, DBA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9400137 A1 (HANSEN, MICHAEL), 6 January 1994 (06.01.94), page 1, lines 22-23; page 4, lines 2-6 --	1-51
X	NEW ZEALAND MEDICAL JOURNAL, Volume 99, August 1986, Robert B Fisher, "Bee venom and chronic inflammatory disease" page 639 - page 640 --	1-51
X	WO 9108753 A1 (GESELLSCHAFT FüR STRAHLEN- UND UMWELTFORSCHUNG MBH (GSF)), 27 June 1991 (27.06.91), claims 35-37 --	1-48,50

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 1999	01-02-1999
Name and mailing address of the ISA/ Swedish Patent Offic Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9003178 A1 (WATANABE, KAZUO), 5 April 1990 (05.04.90), claims 10-20, 48-49; page 1, lines 12-14; page 2, line 4 - page 3, line 8	1-4,7-34, 37-51
A		5-6,35-36
	--	
A	The Medical Letter On Drugs and Therapeutics, Volume 35, No 900, July 1993, C. J. Parkins, "Interferon beta-1B for multiple sclerosis" page 61 - page 64	18-22,41-43
	--	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00448

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-30
   because they relate to subject matter not required to be searched by this Authority, namely:
   Although claims 1-30 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition (c.f. PCT Rule 39.1(iv)).

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest [ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.